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RESEARCH PAPER

Distinct endothelial pathways underlie sexual dimorphism in vascular auto-regulation

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BACKGROUND AND PURPOSE

Pre-menopausal females have a lower incidence of cardiovascular disease compared with age-matched males, implying differences in the mechanisms and pathways regulating vasoactivity. In small arteries, myogenic tone (constriction in response to raised intraluminal pressure) is a major determinant of vascular resistance. Endothelium-derived dilators, particularly NO, tonically moderate myogenic tone and, because the endothelium is an important target for female sex hormones, we investigated whether NO-mediated moderation of myogenic tone differed between the sexes.

EXPERIMENTAL APPROACH

Pressure–diameter or relaxation concentration–response curves to the NO donor spermine-NO or soluble guanylate cyclase (sGC) stimulation (BAY41-2272) were constructed before and following drug intervention in murine mesenteric resistance arteries. Hypotensive responses to activators of the NO-sGC pathway were determined. Quantitative PCR and Western blotting were used for expression analysis.

KEY RESULTS

NO synthase inhibition enhanced myogenic tone of arteries of both sexes while block of endothelium-derived hyperpolarizing factor (EDHF) enhanced responses in arteries of females only. Spermine-NO concentration-dependently relaxed mesenteric arteries isolated from either sex. However, while inhibition of sGC activity attenuated responses of arteries from male mice only, endothelial denudation attenuated responses of arteries from females only. BAY41-2272 and spermine-NO-induced vasodilatation and hypotension were greater in males than in females.

CONCLUSIONS AND IMPLICATIONS

NO moderated myogenic tone in arteries of male mice by a sGC-dependent pathway while EDHF was the predominant endothelial regulator in arteries of females. This is a potentially important sexual dimorphism in NO-mediated reactivity and further implicates EDHF as the predominant endothelial vasodilator in female resistance arteries.

Abbreviations

CVD,cardiovascular disease; EDHF, endothelium-derived hyperpolarizing factor; sGC, soluble guanylyl cyclase; MT, myogenic tone

Introduction

Globally, cardiovascular disease (CVD) is the main cause of death accounting for 29% of all deaths in 2004 (http://www.who.int/). However, it is well established that the incidence of CVD is significantly lower in females compared with age-matched males (Lerner and Kannel, 1986; Barrett-Connor, 1997). Statistics show that this reduced susceptibility to CVD in females is lost following menopause when the rates of CVD become similar in both sexes (Coylewright *et al.*, 2008; Reckelhoff and Maric, 2010) (http://www.BHF.org.uk/). A number of different mechanisms and pathways have been

proposed to explain this sex difference in susceptibility to CVD including the possibility that distinct pathways and mechanisms underlie the control of microvascular tone between the sexes.

The normal tone of the vasculature is regulated by a combination of systemic neuronal and humoral reflexes and localized mechanisms activated by metabolic demand, and physiological forces perturbing the blood vessel wall. This latter local reflex, termed auto-regulation, is described as the innate responsiveness of blood vessels to forces exerted on the vessel wall including that by intraluminal pressure (transmural force that leads to vasoconstriction) and flow (shear stress that leads to vasodilatation) (Davis and Hill, 1999; Chatzizisis *et al.*, 2007; Davies, 2009). Accordingly, aberrant vascular auto-regulation is a complicating factor of several forms of CVD including hypertension (Falcone *et al.*, 1993; Izzard *et al.*, 1996; Dunn *et al.*, 1998), stroke and atherosclerosis (Malek *et al.*, 1999). It is noteworthy that the endothelial cell has a major influence over the extent of vascular auto-regulation, both as the cell that senses shear stress and also via provision of a buffering influence on pressure-induced constriction, that is, in the absence of the endothelium the vasoconstriction induced by elevations of intraluminal pressure is substantially enhanced (Scotland *et al.*, 2001). Interestingly, evidence suggests that sexual dimorphism in auto-regulatory control exists. In particular and of direct relevance to our present findings is the observation that pressure-induced constriction is greater in arteries of males versus females (Wellman *et al.*, 1996; Huang *et al.*, 1997; Skarsgard *et al.*, 1997; Geary *et al.*, 1998; Gros *et al.*, 2002); however, the exact mechanisms involved are uncertain.

Here, we have investigated the possibility that differences in the buffering influence of the endothelium underlie the reduced pressure-induced constriction responses in the resistance vasculature of female compared with male mice. In particular, we show that, in arteries of female mice, the endothelium-derived hyperpolarizing factor (EDHF) plays an important role in moderating pressure-induced constriction but that NO is the predominant endothelium-derived vasodilator moderating myogenic tone (MT) in the resistance vasculature of male mice.

Methods

Animals and tissue collection

All animal care and experimental procedures were conducted according to the Animals (Scientific Procedures) Act 1986, United Kingdom. All studies involving animals are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals (McGrath *et al.*, 2010).

Male and female C57BL/6J mice (8–10 weeks of age; Charles River, Kent, UK; total number = 74) were killed by cervical dislocation and the mesentery removed and placed in cold physiological salt solution (PSS) composed of: mmol·L^{-1}: NaCl (119), KCl (4.7), $CaCl_2 0.2H_2O$ (2.5), $MgSO_4 0.7H_2O$ (1.2), $NaHCO_3$ (25), KH_2PO_4 (1.2) and glucose (5.5). Third-order arteries were isolated and cleared of surrounding fat. Alternatively, for protein or mRNA analysis, whole mesenteric bed was separated from the intestine and snap frozen in liquid nitrogen and then kept at –80°C until use.

To determine the role of female sex hormones in any responses seen, female mice were sham operated or ovariectomized at 4 weeks (Charles River) and then housed until 8–10 weeks of age before collection of tissue for experimentation. Successful ovariectomy was confirmed by measuring plasma oestrogen levels. For blood collection, animals were surgically anaesthetized using 2% isoflurane. Blood was collected by intracardiac puncture in heparin (25 U·mL^{-1} of blood), centrifuged at 13 000 x *g* for 10 min and the plasma collected. Plasma 17β-oestradiol was assessed by a specific enzyme-immunoassay (Cayman Chemicals, Ann Arbor, MI, USA).

Pressure myography

Vessels were mounted in a perfusion myograph continuously superfused with PSS at 10 mL·min^{-1} (37°C, pH 7.4), gassed with 21% O_2/5% CO_2 in N_2, and placed on the stage of an inverted microscope (Nikon, TMS). Vessels were visualized using a video camera (VM-902; Hitachi Denshi Ltd, Tokyo Japan), and the internal diameter determined using a video dimension analyzer (Living Systems Inc, Burlington, VT, USA) and recorded on a PC using Chart 5.1™ software (ADInstruments Ltd., Oxford, UK). After equilibration (1 h), vessels were pressurized to 80 mmHg, allowed to develop spontaneous MT and then the thromboxane A_2-mimetic U46619 (11α, 9β-epoxymethano-PGH_2, 10 nmol·L^{-1}) applied to further constrict vessels followed by exposure to acetylcholine (ACh, 10 μmol·L^{-1}) to test endothelium integrity; vessels not demonstrating spontaneous MT and >50% reversal of U-46619-induced tone in response to ACh were rejected. Vessels were then washed and allowed to equilibrate for a further 45 min before constructing pressure–diameter curves.

Pressure–diameter curves were constructed under no-flow conditions in the absence and then in the presence of specific inhibitors of the three primary endothelial vasodilators. To determine the role of NO or prostacyclin (PGI_2), vessels were superfused with the NOS inhibitor N^G-nitro-L-arginine methyl ester (1H-[1,2,4]-oxadiazolo-[4,3-a]-quinoxalin-1-one (L-NAME); 300 μmol·L^{-1}), the soluble guanylate cyclase (sGC) inhibitor ODQ (1 μmol·L^{-1}; Garthwaite *et al.*, 1995) or the cyclooxygenase inhibitor indomethacin (5 μmol·L^{-1}). Involvement of EDHF was determined using the classical and validated approach (Busse *et al.*, 2002) of the selective blockers of SK_{Ca} (K_{Ca}2.1–2.3) and IK_{Ca} (IK1, K_{Ca}3.1) channels (channel and receptor nomenclature follows Alexander *et al.*, 2011), 1-[(2-chlorophenyl)(diphenyl)methyl]-1 H-pyrazole (TRAM-34, 10 μmol·L^{-1}, Wulff *et al.*, 2000) and apamin (50 nmol·L^{-1}), respectively, which were perfused (10 μL·min^{-1}) through the artery (Doughty *et al.*, 1999). Alternatively, blockers of the smooth muscle pathways involved in EDHF responses were used, that is, barium (30 μmol·L^{-1}; blocker of K_{IR}) and ouabain (1 mmol·L^{-1}; blocker of Na/K ATPase) (Edwards *et al.*, 1998). To investigate the role of the endothelium, 2 mL of air was passed through the vessel 30 min before construction of the second pressure–response curve. The vessel was deemed denuded when there was no subsequent response to bradykinin (300 nmol·L^{-1}). Unless otherwise stated, all inhibitors were superfused over the artery and all for a 30 min pretreatment period. Finally, at the end of each experiment, a third and final pressure–diameter

curve was constructed in the presence of Ca^{2+}-free PSS containing EGTA (2 mmol·L^{-1}) to provide an estimation of the passive diameter at each pressure for calculation of % MT responses.

In some experiments, vasodilator concentration–response curves were constructed, in the absence and then in the presence of inhibitors described above, to the NO donor spermine NO-NOate (SPER-NO; 10 nmol·L^{-1}–10 μmol·L^{-1}) or sGC activator, BAY41-2272 (10 nmol·L^{-1}–10 μmol·L^{-1}; Koglin *et al.*, 2002; Stasch *et al.*, 2002), superfused into the organ chamber in vessels pressurized to 100 mmHg.

Quantitative RT-PCR (q-PCR) of murine mesenteric tissue

Samples were homogenized and RNA extracted for quantitative real-time PCR (qRT-PCR) analysis with SYBR green, using specifically designed primers for each gene of interest:

SK1- 5′-GTGAAGATTGAACAAGGGAAGG-3′ and 5′-TGCC TCCAACTCCTCCTG-3′;

SK2- 5′-ACCATCAGACAGCAGCAAAGGG-3′ and 5′-GAC CGCCGCCTCCTGGAC-3′;

SK3- 5′-GCCAACTCTACCGCCATC-3′ and 5′-GGCTG TGGAACTTGGAGAG-3′;

IK1- 5′-ATGCTCCTGCGTCTCTAC-3′ and 5′-GAAGCGG ACTTGGTTGAG-3′;

Cx37- 5′-AGGCAGGCTTCCTCTATGGC-3′ and 5′-AGACA TAGCAGTCCACGATGTG-3′;

Cx40- 5′-GAGGCCCACGGAGAAGAATG-3′ and 5′-TGG TAGAGTTCAGCCAGGCT-3′;

Cx45-5′-CGGGCTGTGAGAATGTCTGC-3′ and 5′-CAGG TACATCACAGAGGGAGTTG-3′;

sGCα1- 5′-ACACTCGCTTTGACCAGCA-3′ and 5′-CAATAT GCATCCCCGATGG-3′;

sGCβ1- 5′- TCAGTGTGGCAATGCCATC-3′ and 5′- GCG GACCAGAGAGAAGACAGA-3′.

qRT-PCR was performed using an ABI Prism 7900 sequence detection system. Expression of each gene was normalized to 18-S, 5′-AGCCTGCGGCTTAATTTGAC-3′ and 5′-CAACTAAGAACGGCCATGCA-3′, and expressed as a relative value using the comparative threshold cycle (Ct) method ($2^{-\Delta\Delta Ct}$) (Livak and Schmittgen, 2001). The levels of mRNA were expressed in females relative to males, and in ovariectomized relative to sham-operated animals.

Western blotting

Mesenteric vascular beds were homogenized in ice-cold phospho-homogenization buffer (mmol·L^{-1}: Tris 10, NaCl 50, $NaPP_i$ 30, EDTA 2, NaF 50, PMSF 1, Na_3VO_4 1; 10 μg·mL^{-1} protease inhibitors benzamidine, antipain, leupeptin, and aprotinin, and 1% Triton X-100) using the Precellys™ bead grinder homogenizer. Samples were centrifuged at 13 000 x *g* for 15 min at 4°C and the supernatant was retained. Equal amounts of protein (20 μg) and KCa3.1 positive control of COLO 320 DM cell lysate (25 μg; Santa Cruz Biotechnology, Santa Cruz, CA, USA) were subjected to 7.5% SDS gel electrophoresis under reducing conditions. Separated proteins were then electrotransferred onto nitrocellulose membrane (GE Healthcare, Amersham, UK) and incubated overnight at 4°C with primary antibody goat anti-human $K_{Ca}3.1$ (1:1000; IK_{Ca}; Santa Cruz Biotechnology) or anti-actin (1:20 000; Millipore, Billerica, MA, USA). Membranes were then incubated with horseradish peroxidase conjugated secondary antibody (rabbit anti-goat or goat anti-mouse, respectively, 1:1000, Dako, Glostrup, Denmark) and the antibody-protein complexes were visualized using an enhanced chemiluminescence (ECL™) detection system (LumiGLO™ Reagent and Peroxide, Cell Signaling Technology™, UK) and autoradiographic film (Hyperfilm, Amersham Biosciences, UK). Densitometric analysis was performed with Scion Image 4.0.3 Gel Analyzer software (National Institues of Health, Bethesda, MD, USA). The levels of protein were expressed relative to α-actin expression.

BP measurements

Mice were maintained under anaesthesia (isoflurane, 1.5%, in 100% O_2 at 0.4 L·min^{-1}) throughout the experiment and body temperature was kept constant at 37.5°C. Catheters (polyvinyl tubing, 0.61 mm outside diameter) were placed in the carotid artery and jugular vein for arterial BP measurements and intravenous injections of vasodilators. BAY41-2772 (100–300 μg·kg^{-1} in 5% DMSO), SPER-NO (1–10 μg·kg^{-1} in saline) and vehicles were administered into the jugular vein in 50 μL bolus doses using a 0.5 mL insulin syringe. BP was evaluated throughout.

Data analysis

All values are expressed as the arithmetic mean ± SEM. Pressure-induced constriction is expressed as %MT, where $\%MT = 100(D_{Passive} - D_{Active}/D_{Passive})\%$. Statistical analysis was performed using paired or unpaired Student's *t*-test for two groups and using two-way ANOVA for comparison of curves followed by Dunnett's test for comparison to baseline or Bonferroni post-tests for comparison of specific groups. Differences were considered significant at $P < 0.05$. All *n* values equally represent the number of experiments conducted and animals used.

Results

Arteries of female mice express less pressure-induced constriction than arteries of male mice

As previously reported (Gros *et al.*, 2002), Ca^{2+}-dependent MT was of greater magnitude and first evident at lower pressures (60 vs. 80 mmHg respectively) in arteries of male compared with female mice [($P < 0.001$) Figure 1A and B]. There were no differences in basal vessel diameter, passive diameter and wall thickness of third-order arteries between the sexes (Supporting Information Table S1).

Ovariectomy significantly ($P < 0.001$) reduced plasma oestradiol concentration (Sham: 63.6 ± 6.7 pg·mL^{-1}; OVX: 21.0 ± 4.3 pg·mL^{-1}). Ovariectomy did not alter basic blood vessel structural parameters (Supporting Information Table S2) in arteries of sham-operated ($n = 22$) and ovariectomized ($n = 17$) animals respectively. However, MT was enhanced in arteries of ovariectomized compared with sham-operated mice ($P < 0.05$, Figure 1C), with significant differences between the groups evident at pressures of 80 and 100 mmHg.

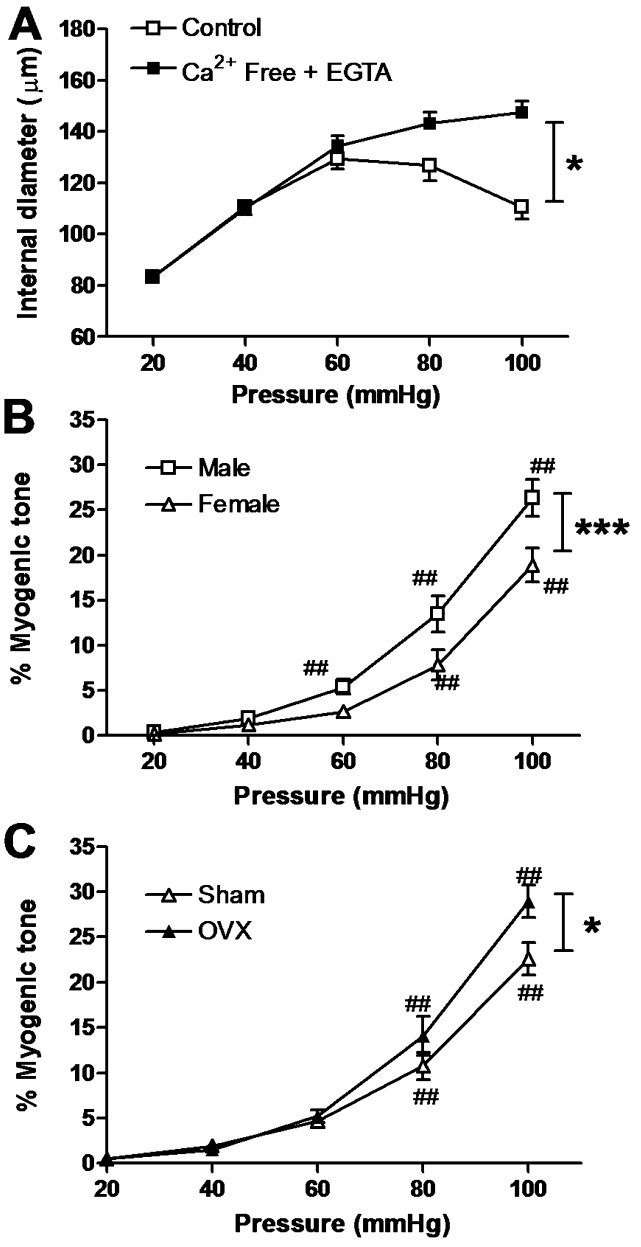

Figure 1

Intraluminal pressure–diameter relationship in mesenteric small arteries from (A) male (n = 17) wild-type (WT) mice in the absence and presence of Ca^{2+} free PSS + EGTA (2 mmol·L^{-1}) and (B) myogenic response curves in mesenteric arteries of male (n = 17) and female (n = 19) and in (C) sham-operated (n = 22) and ovariectomized (OVX: n = 17) WT mice. Data are shown as the arithmetic mean ± SEM. *P < 0.05, ***P < 0.001, significantly different as shown; two-way ANOVA. ##P < 0.01, significantly different from % myogenic tone at 20 mmHg; Dunnett's post-tests.

Up-regulation of the EDHF pathway, but not NO or PGI_2, in females underlies reduced pressure-induced constriction in resistance arteries

EDHF blockade significantly enhanced MT in arteries of female (P < 0.05, n = 6), but not male (P > 0.05, n = 7; Figure 2A and B) or ovariectomized mice (n = 7; Figure 2C and D). Importantly, application of TRAM-34 alone to female arteries likewise enhanced pressure-induced constriction (P < 0.01, Figure 2E). Consistent with these observations, the expression of mRNA for $K_{Ca}3.1$ (IK1) channels was enhanced 14-fold in mesenteric tissue of females, compared with male mice (P < 0.001, Figure 2F). In addition, ovariectomy caused a sixfold decrease in levels compared with sham controls implicating female sex hormones, at least in part, in these differences (Figure 2G). The differences in $K_{Ca}3.1$ mRNA were similarly reflected in protein levels (Figure 2H). In contrast, there were no significant differences in the SK1, SK2, SK3, Cx37, Cx40 and Cx45 genes that have also been implicated in the EDHF pathway (Supporting Information Figures S1 and S2).

L-NAME enhanced MT in arteries of both male (P < 0.05, n = 5) and female (P < 0.01, n = 6) mice (Figure 3A and B), as previously reported, and both sham-operated (P < 0.05, n = 10) and ovariectomized (P < 0.01, n = 5) mice (Figure 3C and D). In contrast, indomethacin had no effect in any vessels studied (n = 6–7, Supporting Information Figure S3). Interestingly, combination of Tram-34 + apamin treatment with L-NAME in arteries of female mice produced no greater enhancement of MT compared with vessels treated with Tram-34 + apamin alone (%MT at 100 mmHg of 30.2 ± 4.0 vs. 32.3 ± 7.4% respectively; n = 4, P > 0.05).

NO and sGC-dependent relaxation

Because the combination of NOS inhibition and EDHF blockade produced no greater effect than EDHF blockade alone, we investigated the possibility that the pathways by which NO moderates MT might be different between the sexes. Indeed, inhibition of sGC activity using ODQ enhanced responses to pressure in arteries of male (P < 0.05) but not female mice (Figure 4A and B). To investigate this difference more closely, we next investigated the effect of ODQ on NO donor-induced relaxation of pressurized arteries. Interestingly, while ODQ attenuated SPER-NO-induced vasodilatation of arteries of male mice (P < 0.01, n = 6, Figure 4C), it had little effect on responses in arteries of female mice (n = 6, Figure 4D).

In accord with this difference in NO function, sGCα1 and β1 mRNA levels were greater in mesenteric tissue of male compared with female mice (Figure 4E and F) and in ovariectomized compared with sham-operated (Figure 4G and H) mice. Furthermore, in line with this observation, the sGC activator, BAY41-2272, caused substantially greater vasodilatation of arteries isolated from male mice compared with those from females (Figure 5A and B). This difference in isolated vessels *in vitro* translated *in vivo* with the demonstration that BAY41-2272 caused greater decreases in BP in male compared with age-matched female mice (Figure 5C). Similarly, the NO donor, SPER-NO, decreased BP in both sexes with no differences between the sexes (Figure 5D).

NO-induced relaxation of murine resistance arteries of female mice is endothelium dependent and involves EDHF activity

Endothelium denudation suppressed the relaxation, induced by SPER-NO, of resistance arteries of female but not male mice (Figure 6A and B). This endothelial dependency was

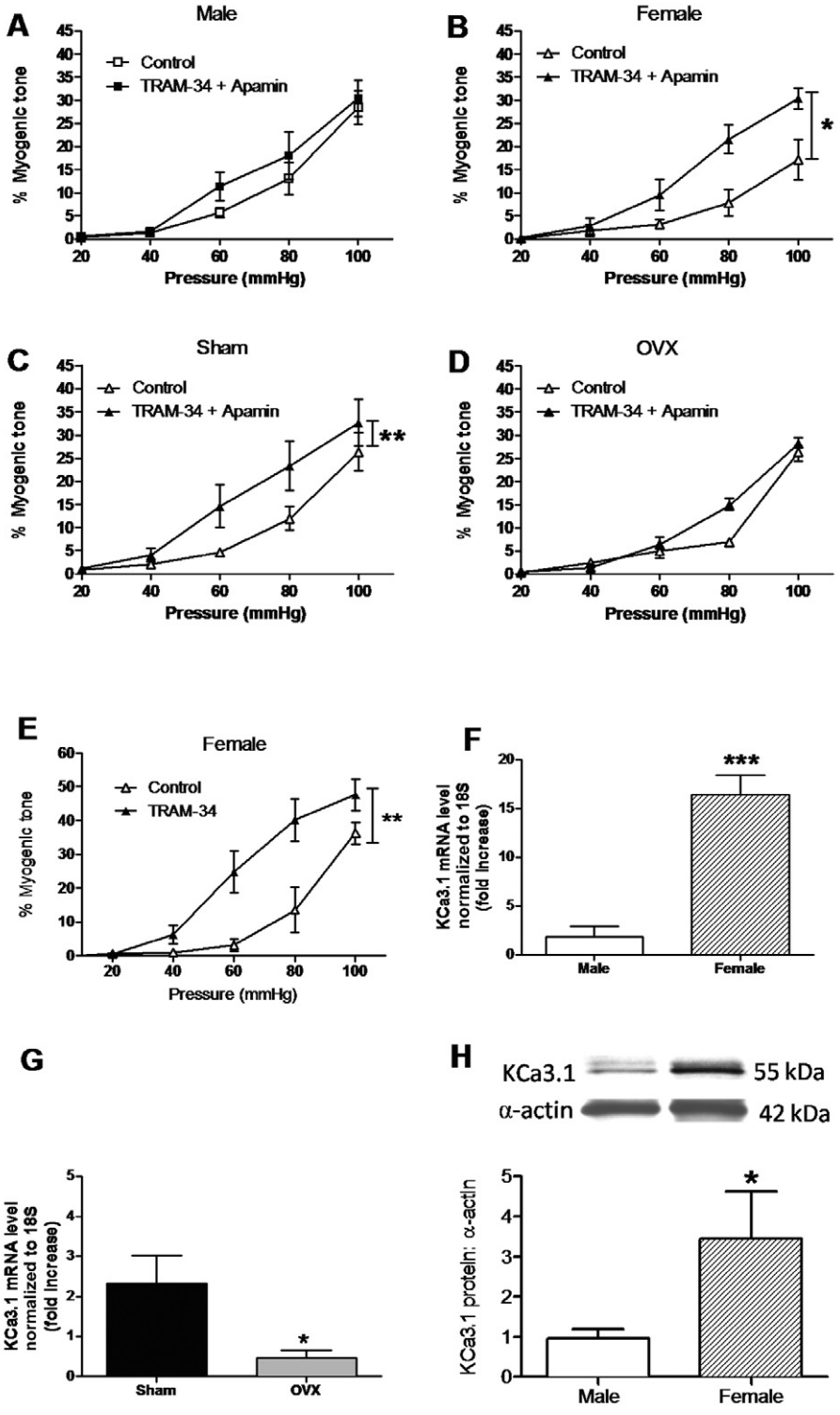

Figure 2

Pressure-induced constriction (% myogenic tone) of mesenteric arteries of (A) male (n = 7), (B) female (n = 6), (C) sham-operated (n = 6) and (D) ovariectomized (n = 7) mice in the absence and presence of TRAM-34 (10 μmol·L^{-1}) + apamin (50 nmol·L^{-1}) and (E) in the presence of TRAM-34 (10 μmol·L^{-1}, n = 6) alone. (F) mRNA expression for K_{Ca}3.1 channels in mesenteric tissue of male (n = 5) and female (n = 5) and (G) protein expression for K_{Ca}3.1 channels in mesenteric tissue of male (n = 8) and female (n = 6) mice. (H) mRNA expression for K_{Ca}3.1 channels in mesenteric tissue of sham-operated (n = 4) and ovariectomized (OVX; n = 4) mice. The mRNA data are expressed as fold increase above mean values in male or sham-operated females and protein expression relative to α-actin. Data are shown as the arithmetic mean ± SEM. *P < 0.05, **P < 0.01, ***P < 0.001, significant differences between curves or groups; two-way ANOVA or a Student's unpaired t-test.

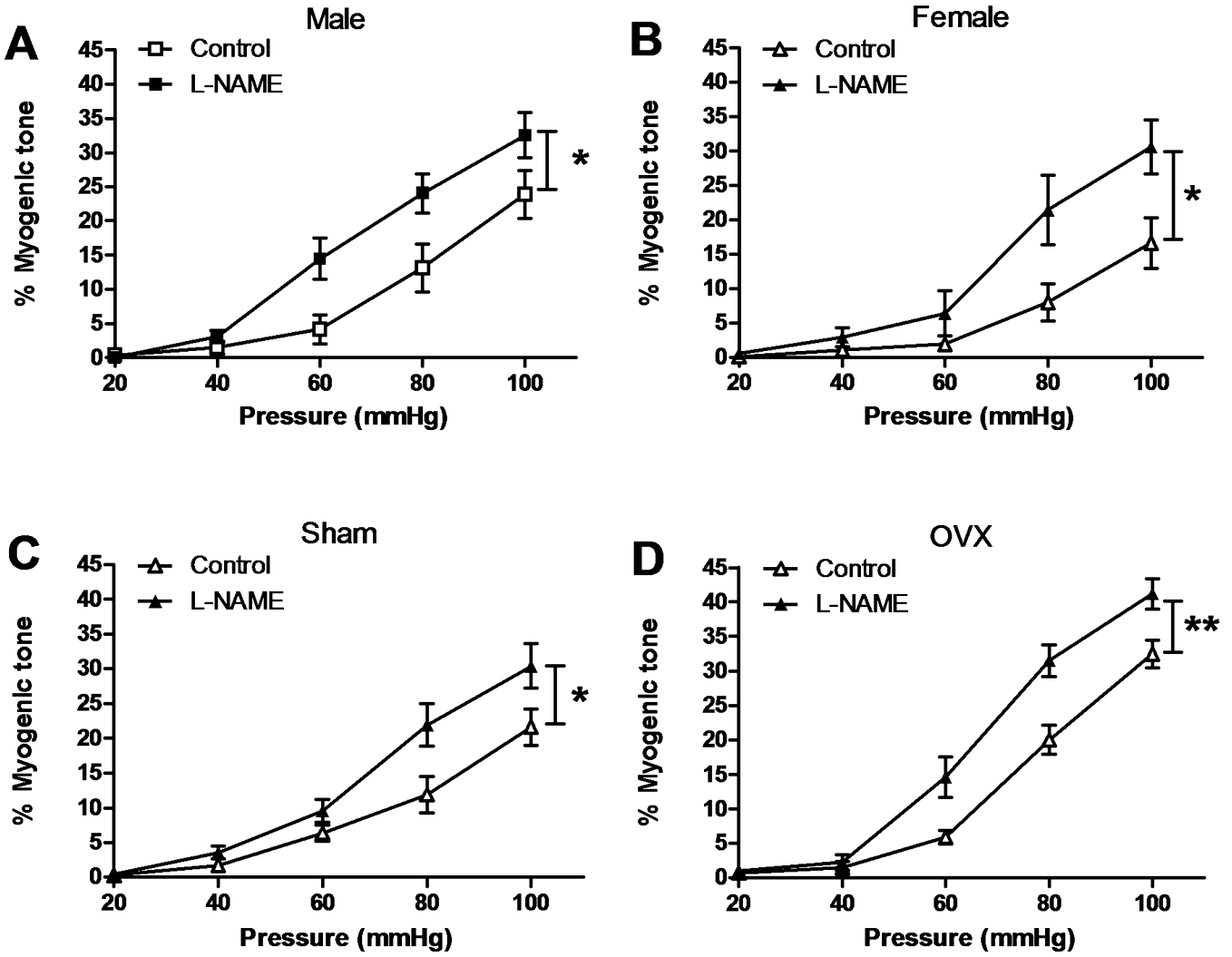

Figure 3

Pressure-induced constriction of mesenteric arteries of (A) male (n = 5), (B) female (n = 6), (C) sham-operated (n = 10) and (D) ovariectomized (n = 5) C57BL/6J mice in the absence and presence of L-NAME (300 μmol·L^{-1}). Data are shown as the arithmetic mean ± SEM. *P < 0.05, **P < 0.01, significant differences between curves; two-way ANOVA.

likely to be related to differences in EDHF activity because EDHF blockade with TRAM-34 and apamin (Figure 6C and D) or barium and ouabain (Figure 6E and F) both profoundly suppressed NO-mediated vasodilatation in arteries of female but not male mice. Treatment of arteries from female mice with TRAM-34 alone also significantly suppressed SPER-NO-induced relaxation (Figure 6G).

Discussion and conclusions

Intraluminal pressure-induced constriction is a major determinant of peripheral resistance, acting as an opposing influence to flow-mediated dilatation. Together, these phenomena play an important role in determining organ blood flow and BP. In the present study, we demonstrated that a female sex hormone-mediated suppression of MT was associated with an enhanced release of EDHF in small mesenteric arteries of female mice. Moreover, this enhancement was likely to be due to the selective up-regulation of the K_{Ca}3.1 channel, which is an essential component of the EDHF release pathway. We speculate that this selective EDHF-mediated suppression of pressure-induced constriction contributes to the improved vascular reactivity that is thought to underlie the relative protection of females from CVD.

Elevation of intraluminal pressure in murine mesenteric arteries evoked a pressure-dependent constriction in arteries of both sexes that was abolished in the absence of calcium as demonstrated previously (Knot and Nelson, 1998). In addition, the magnitude of this response in arteries of male mice was significantly greater than that evident in age-matched female animals, in agreement with previous findings in small arteries of various species (including humans) throughout the vasculature including coronary (Wellman *et al.*, 1996; Miller,

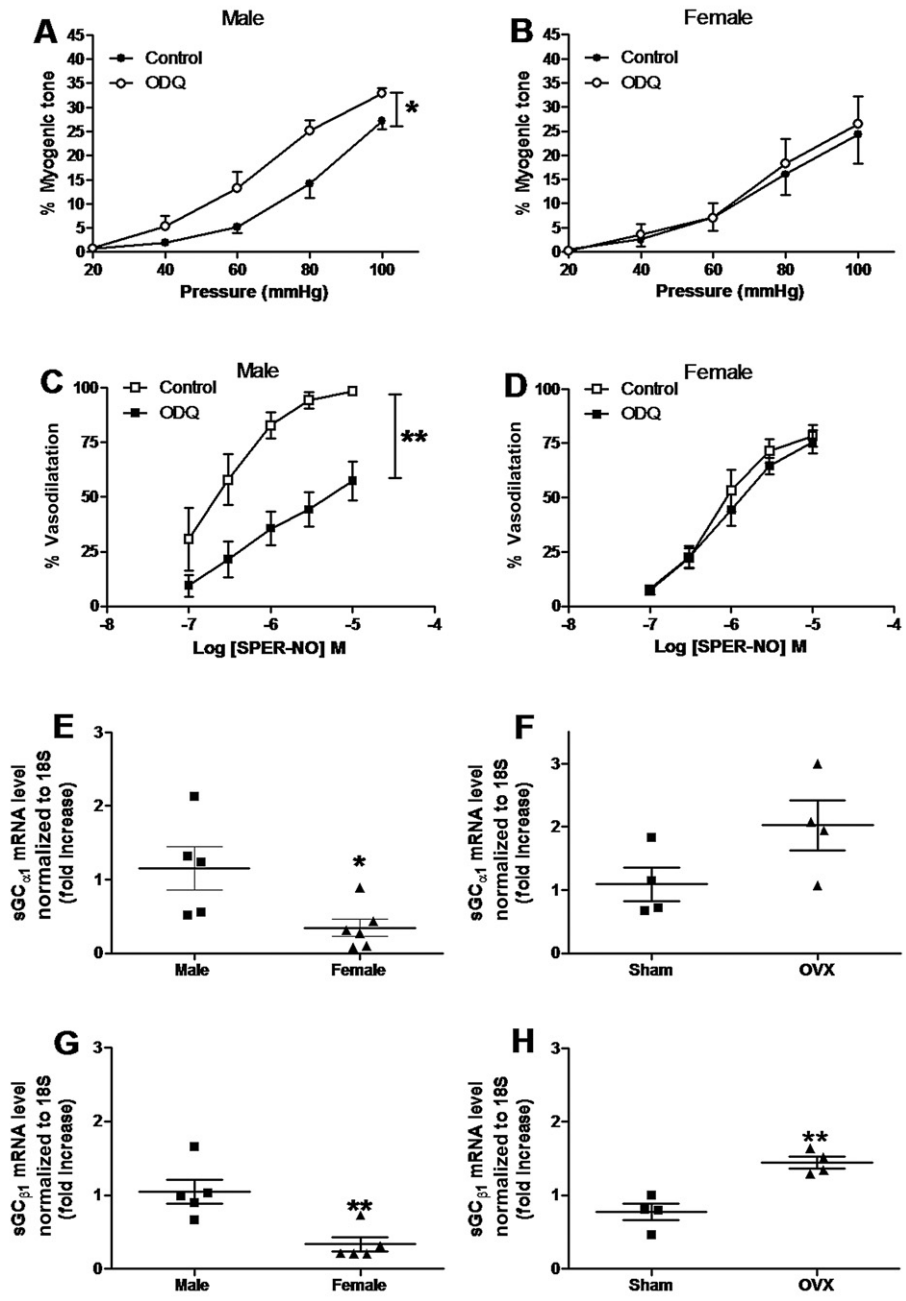

Figure 4

Pressure-induced constriction (% myogenic tone) of mesenteric arteries of (A) male ($n = 5$), (B) female ($n = 4$) and SPER-NO-induced vasodilatation of (C) male ($n = 6$) and (D) female ($n = 6$) wild-type mice in the absence and presence of ODQ (1 μmol·L^{-1}). mRNA expression for sGCα1 in mesenteric tissue of (E) female ($n = 5$) and male ($n = 5$) and (F) sham-operated ($n = 6$) and ovariectomized (OVX; $n = 7$) mice and mRNA expression for sGCβ1 in mesenteric tissue of (G) female ($n = 5$) and male ($n = 5$) and (H) sham-operated ($n = 7$) and ovariectomized (OVX; $n = 9$) mice. The mRNA data are expressed as fold increase above mean values in male or sham-operated females. Data are shown as the arithmetic mean ± SEM. *$P < 0.05$, **$P < 0.01$, significant differences between curves; two-way ANOVA.

Jr. *et al.*, 1997), cerebral (Skarsgard *et al.*, 1997; Geary *et al.*, 1998), gracilis muscle (Huang *et al.*, 1997), tail (Pak *et al.*, 2002) and mesenteric arteries (Gros *et al.*, 2002). These differences in reactivity were not due to differences in structural characteristics of the vessels, as wall thickness and passive diameter remained unchanged (parameters that most closely reflect remodelling of resistance arteries) (Mulvany *et al.*, 1996; Martinez-Lemus *et al.*, 2009), suggesting that remodelling is unlikely to underlie the sex differences observed. The widespread expression of this sex difference throughout the vasculature indicates that the phenomenon is not organ specific but is likely to be a critical difference that underlies the

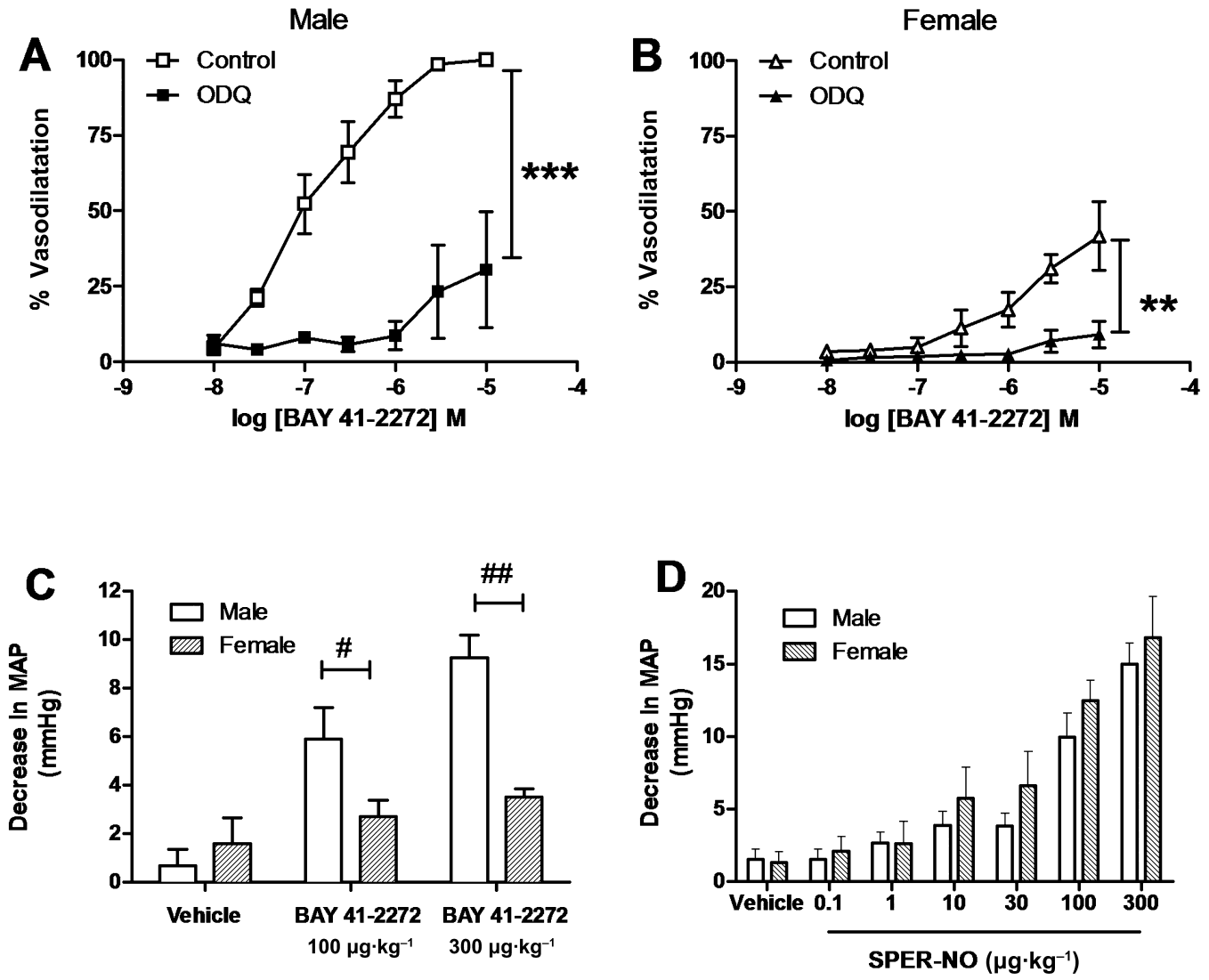

Figure 5

BAY 41-2272-induced vasodilatation of (A) male ($n = 4$) and (B) female ($n = 4$) wild-type mice in the absence and presence of ODQ (1 µmol·L^{-1}). (C) Decrease in BP (as mean arterial pressure; MAP) in male ($n = 5$–9) and female ($n = 4$–9) C57/BL6 mice after the infusion of BAY 41-2272 (100 µg·kg^{-1}) or (D) SPER-NO (1–10 µg·kg^{-1}). Data are shown as the arithmetic mean ± SEM. $^{**}P < 0.01$ and $^{***}P < 0.001$, significant differences between curves; two-way ANOVA. BP data analysed using two-way ANOVA followed by Bonferroni post-tests are shown as $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$, significant differences as indicated.

major differences in vascular reactivity between the sexes. The rather generic expression of this phenomenon including within the mesenteric vasculature provides a robust justification for the use of mesenteric arteries that are relatively much easier to isolate without causing damage than other resistance arteries, for further mechanistic investigation. In addition to increased MT, arteries of male mice exhibited an enhanced sensitivity to pressure, shown by the fact that significant MT was evident at lower intraluminal pressures (i.e. 60 mmHg) compared with arteries of female animals (i.e. 80 mmHg); an observation also in agreement with previous findings in mesenteric vessels of mice (Gros *et al.*, 2002). It is likely that female sex hormones, at least in part, underlie this sexual dimorphism as ovariectomy of mice resulted in elevated responses to pressure compared with sham controls, although no difference in the sensitivity to pressure was evident. Because the baseline characteristics of the arteries were not significantly different between the sham and ovariectomized mice, the differences in MT are unlikely to be due to structural changes.

As the endothelium has been identified as providing an opposing buffering influence over myogenic constriction in various blood vessels including mouse mesenteric resistance arteries (Scotland *et al.*, 2001), we investigated the possibility that sexual diversity in this endothelial influence might underlie our observations. While pressure-induced constriction was evident in mesenteric arteries of both sexes, we identified a clear distinction between the sexes in the oppos-

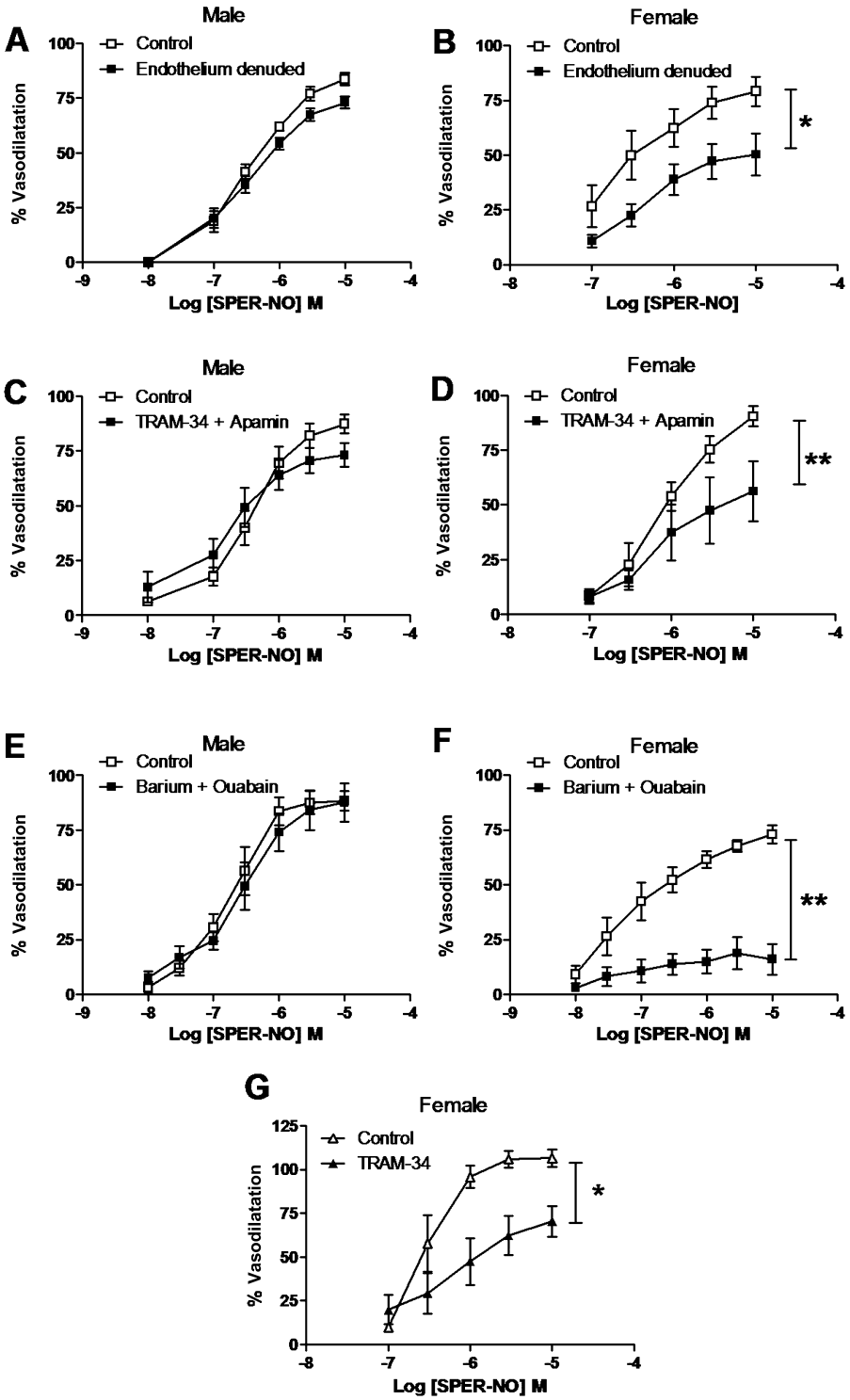

Figure 6

SPER-NO-induced vasodilatation of (A) male (n = 6) and (B) female (n = 7) mice before and after endothelium denudation (2 mL air), and of (C) male (n = 8) and (D) female (n = 5) mice in the absence and presence of TRAM-34 (10 μmol·L^{-1}) + apamin (50 nmol·L^{-1}) and of (E) male (n = 5) and (F) female (n = 5) mice in the absence and presence of barium (30 μmol·L^{-1}) + ouabain (1 mmol·L^{-1}) and of (G) female mice (n = 5) in the absence and presence of TRAM-34 (10 μmol·L^{-1}) alone. Data are shown as the arithmetic mean ± SEM. *P < 0.05, **P < 0.01, significant differences between curves; two-way ANOVA or Student's unpaired t-test.

ing endothelial pathways employed to moderate the extent of this contractile response. Indeed, in arteries of female mice, blockade of the EDHF pathway, achieved via combined blockade of IK_{Ca}, through $K_{Ca}3.1$ and SK_{Ca}, channels, considered the hallmark of an EDHF response (Busse *et al.*, 2002), substantially enhanced MT while these agents had little effect in arteries of male mice. This finding correlates well with previous observations that endothelium-dependent vasodilatation of resistance arteries appears to be predominantly mediated by EDHF in females; an observation noted in murine mesenteric arteries by us previously (Scotland *et al.*, 2005) in rat mesenteric arteries by others (McCulloch and Randall, 1998), but also in other arteries including rat tail artery and extra- and intra-vaginal resistance arteries (Morton *et al.*, 2007). These observations also fit well with our previous reports demonstrating that systemic BP in male mice deficient in both eNOS and COX-1 is significantly elevated, whereas females of the same genotype are normotensive (Scotland *et al.*, 2005); highlighting a pivotal role for EDHF in governing peripheral resistance in females.

Our observations also suggest that this enhanced role for EDHF is in part mediated by female sex hormones as blockade of EDHF pathways in arteries taken from ovariectomized mice had a substantially reduced effect, that is, treatment with Tram-34 + apamin only caused a minor but non-significant enhancement of pressure-induced constriction. Other studies investigating EDHF responses suggest that oestrogen is likely to be responsible for this effect on EDHF as vasorelaxant responses to endothelium-dependent vasodilators are lost in arteries of ovariectomized female rats (Liu *et al.*, 2002; Chataigneau and Schini-Kerth, 2005; Nawate *et al.*, 2005; Burger *et al.*, 2009) but restored following oestrogen replacement or in mice deficient in oestrogen β receptors (Luksha *et al.*, 2006).

The mechanism by which the propagation of the EDHF responses in females is enhanced has remained uncertain. However, here we have identified a profound increase in the expression of mRNA and protein for $K_{Ca}3.1$ channels in arteries of female compared with male mice, as well as a sixfold decrease in mRNA levels following ovariectomy. These findings suggest that the enhanced EDHF reactivity evident in mesenteric arteries from females is likely to be due to an augmenting effect of female sex hormones on expression of $K_{Ca}3.1$ channels. It is likely that this expression is occurring within the endothelium as studies show localization of this channel exclusively to the endothelium of rat and murine blood vessels (Edwards *et al.*, 1998; Doughty *et al.*, 1999; Walker *et al.*, 2001; Brahler *et al.*, 2009) and human endothelial cells of mesenteric arteries (Kohler *et al.*, 2000). In support of the concept that oestrogens influence $K_{Ca}3.1$ channels, recent studies in the human sweat gland epithelial cell line NCL-SG3 have shown that oestrogen treatment rapidly activates a whole cell K^+ current mediated through $K_{Ca}3.1$ channels that is independent of oestrogen receptor activation and a consequence of the rapid translocation of $K_{Ca}3.1$ channels to the cell membrane (Muchekehu and Harvey, 2009). Whether such translocation might also underlie the female sex hormone-dependent effects in the present study is uncertain and warrants investigation. There is also evidence suggesting that myoendothelial gap junction protein, connexin 43, is up-regulated by oestrogen treatment (Liu *et al.*, 2001; Nawate *et al.*, 2005). However, we found no significant differences in the mRNA expression of connexins 45, 43, 40 or between the sexes or between sham and ovariectomized females. Because we observed only significant changes in $K_{Ca}3.1$ channels and no differences between the sexes in the expression of the SK_{Ca} channels, we explored the possibility that the former channel might be solely responsible for the effects of EDHF blockade seen in our studies. Exposure of arteries from female mice to TRAM-34 alone substantially enhanced pressure-induced constriction, exerting an effect almost identical in magnitude to that evident following treatment with TRAM-34 + apamin. This surprising result suggests that in mesenteric arteries of female mice, activation of $K_{Ca}3.1$ channels alone is sufficient to trigger the EDHF phenomenon. This is at odds with a number of previously published observations indicating close dependency and co-operativity between the SK_{Ca} and IK_{Ca} channels to bring about EDHF-dependent responses (Busse *et al.*, 2002; Crane *et al.*, 2003; Villar *et al.*, 2007). The exact reason for the apparent differences between our present study and others is uncertain but might relate to the fact that all previous studies describing the co-operativity between the endothelial channels were conducted in arteries of male mice.

The present study also highlights a sex difference in the pathway for NO-induced vasodilatation in mesenteric arteries. Blockade of NO synthesis resulted in enhanced constrictor responses to pressures inclusive of 40–100 mmHg in arteries of both sexes to a similar degree (with an approximate doubling of responses). This finding is in accord with previous studies in mesenteric (Nguyen *et al.*, 1999; Scotland *et al.*, 2001; Veerareddy *et al.*, 2004) and other artery types (De Wit *et al.*, 1999; Veerareddy *et al.*, 2002) demonstrating a relatively ubiquitous role for NO in modulation of MT. However, in arteries of male mice while NO-induced vasodilatation appeared entirely dependent upon sGC activation, and presumably cGMP elevation, this was not the case in arteries of female mice. A potential explanation for this finding is that the sexes express differential levels of sGC. Consistent with this possibility is our observation of significantly raised levels of the sGCα1 and β1 subunits in mesenteric tissue of males compared with females. The reduced levels of sGC in females are reflected, and indeed supported, by our studies demonstrating substantially reduced effects of the sGC activator, BAY 41–2772, on vascular tone and BP in females compared with males. It is likely that female sex hormones, in part, mediate this difference, as ovariectomy of female mice resulted in a significant elevation of the levels of both sGC subunits. This effect may relate specifically to the activity of oestrogen as previous studies in rats demonstrate oestrogen-induced suppression of sGC expression (Krumenacker *et al.*, 2001). Our findings might also provide some further insight into studies with sGCα1 knockout mice demonstrating that while the male knockouts are hypertensive the females are not (Buys *et al.*, 2008).

Intriguingly, despite the reduced role of sGC in females, in keeping with previous work, our studies demonstrate a similar and substantial role for NO in moderation of pressure-induced constriction, and potent effects of NO donors in both mesenteric resistance arteries and BP in both sexes. The explanation for this apparent paradox, we believe, is that while NO-mediated vasodilatation operates in part via the

classical NO-sGC-cGMP pathway in females, in addition, other sGC-independent pathways for NO activity exist. Furthermore, it is also possible that the role of these distinct pathways in females depends largely upon the type of stimulus, that is, via circulating hormones or shear stress (where cGMP has been implicated in NO-mediated responses) versus transmural pressure as in the present study. In particular, our data support a role for NO-mediated stimulation of EDHF in pressurized arteries of female mice as responses to the NO donor SPER-NO were significantly attenuated in endothelium-denuded arteries or in arteries treated with blockers of the EDHF pathway. In addition, while blockade of EDHF activity or NO generation equally enhanced MT, the combination of the two produced no greater effect than either alone suggesting a convergence of the pathways involved in the enhancing effects on MT in females of these interventions. This finding is in contrast to previous studies (Bauersachs *et al.*, 1996) suggesting that NO inhibits EDHF release in blood vessels; an observation supported by our own findings in eNOS knockout mice demonstrating some up-regulation of EDHF pathways in the absence of NO (Scotland *et al.*, 2001). However, a possible explanation for this apparent discrepancy is that these previous studies were conducted in blood vessels of male animals and a comparison with arteries of female animals was not made. It is also noteworthy that the effects of the NO donor in isolated arteries appear to be greater in males than females, although no differences were evident in the effects upon BP. The exact reason for this difference between the *in vivo* and *in vitro* studies is uncertain and further investigation of this issue is warranted.

Our findings also provide a possible explanation for earlier findings in isolated vessels of NO-induced hyperpolarization and relaxation attenuated by K^+ channel blockade. Our data suggest that, in part, this sensitivity may reflect inhibition of EDHF activity as well as direct smooth muscle cell hyperpolarization (see Vanheel and Vand, 2000). It is noteworthy that endothelium denudation did not alter SPER-NO-induced vasodilatation in arteries of male mice. These findings are consistent with recent observations in male mice with a smooth muscle specific deletion of the β1 subunit of sGC where NO-induced responses were completely abolished (Groneberg *et al.*, 2010).

In summary, this study has shown that while NO plays an important modulatory role in opposing the pressure-induced constriction in both sexes, EDHF plays a major role in the female microcirculation under physiological conditions. Our findings together with recent observations implicating EDHF-dependent pathways in repression of vascular inflammation (Villar *et al.*, 2011) lend further support to the proposal that EDHF plays an important role in mediating vasoprotection in females.

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Conflicts of interest

None.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Figure S1 mRNA expression for SK_{Ca} channel isoforms SK1, SK2 and SK3 in mesenteric tissue of (A, C, E) female (n = 5) and male (n = 5) and (B, D, F) sham-operated (n = 5) and ovariectomized (n = 5) C57BL/6J mice. The data are expressed as fold increase above mean values in male or sham-operated females. There were no significant differences using unpaired Students t-test.

Figure S2 mRNA expression for connexin isoforms 37, 40, 43 and 45 in mesenteric tissue of (A, C, E, G) female (n = 5) and male (n = 5) and (B, D, F, H) sham-operated (n = 5) and ovariectomized (n = 5) C57BL/6J mice. The data are expressed as fold increase above mean values in male or sham-operated females. There were no significant differences using unpaired Students t-test.

Figure S3 Pressure-induced constriction of mesenteric arteries of (A) male (n = 5), (B) female (n = 7), (C) sham-operated (n = 6) and (D) ovariectomized (n = 5) C57BL/6J mice in the absence (open symbols) and presence (closed symbols) of indomethacin (5 $\mu mol \cdot L^{-1}$). Data are shown as the arithmetic mean ± SEM. No significant differences between curves using two-way ANOVA.

Table S1 Comparison of baseline parameters between male and female wild-type mice. ACh (10 $\mu mol \cdot L^{-1}$) response was calculated as $100(D_{ACh} - D_{U\text{-}46619}/D_{80mmHg} - D_{U\text{-}46619})\%$ where D is diameter of the vessel. Values are shown as the arithmetic mean ± SEM. No significant difference is shown as n.s. $P > 0.05$. N values indicate the number of animals.

Table S2 Comparison of the structural parameters between sham-operated and ovariectomized wild-type mice. Values are shown as the arithmetic mean ± SEM. No significant difference is shown as n.s. $P > 0.05$. N values indicate the number of animals.

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